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Enhanced susceptibility of hybrid tilapia to *Flavobacterium columnare* after parasitism by *Ichthyophthirius multifiliis*

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ABSTRACT

Bacterium *Flavobacterium columnare* and protozoan *Ichthyophthirius multifiliis* are two common pathogens of cultured fish. The objective of this study was to evaluate the susceptibility of hybrid tilapia (*Oreochromis* spp.) to *F. columnare*, including fish mortality and bacterial loads in fish gill and kidney after parasitism by *I. multifiliis*. Fish received the following treatments: 1) non-infected control; 2) infected by *I. multifiliis* at 30,000 theronts fish^{-1} alone; 3) infected by *F. columnare* ALM-05-53 at 4.59×10^7 CFU mL^{-1} alone; 4) infected by *I. multifiliis* at 30,000 theronts fish^{-1} and exposed to *F. columnare* ALM-05-53 at 4.59×10^7 CFU mL^{-1}; 5) infected by *F. columnare* TN-3-2012 at 4.27×10^7 CFU mL^{-1} alone; and 6) infected by *I. multifiliis* at 30,000 theronts fish^{-1} and exposed to *F. columnare* TN-3-2012 at 4.27×10^7 CFU mL^{-1}. *F. columnare* in fish tissues were quantified by quantitative real-time polymerase chain reaction and reported as genome equivalents per mg of tissue (GEs mg^{-1}). The results demonstrated that the *I. multifiliis*-parasitized tilapia showed significantly ($P < 0.05$) higher mortality (60.4%) when exposed to *F. columnare* ALM-05-53 than non-parasitized fish (29.1%). The bacterial loads of *F. columnare* ALM-05-53 in fish infected by 30,000 theronts fish^{-1} were ≥5703 GEs mg^{-1} which was between 13 and 17 fold higher than those of non-parasitized fish (≤472 GEs mg^{-1}). Similarly, parasitized tilapia showed significantly higher mortality (25%) and bacterial loads (≥1586 GEs mg^{-1}) at day 3 post-exposure to *F. columnare* TN-3-2012 than non-parasitized fish (0% and ≤197 GEs mg^{-1}). *I. multifiliis* parasitism of tilapia enhanced *F. columnare* invasion and resulted in higher fish mortality.

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1. Introduction

Ichthyophthirius multifiliis Fouquet is a serious protozoan parasite and epizootics have been reported in various freshwater fishes worldwide (Hines and Spira, 1974). The parasite damages fish gills and skin, results in high fish mortality, and leads to substantial economic losses for aquaculture (Traxler et al., 1998). The life stages of the parasite include an infective theront, a parasitic trophont and a reproductive tomont (Dickerson, 2012; Matthews, 2005).

Flavobacterium columnare, causative agent of columnaris, is a Gram-negative bacterium, affecting many commercially important freshwater fish worldwide and may also result in high fish mortality (Declercq et al., 2013). Columnaris is generally regarded as an external infection of fish with clinical signs of skin lesions, fin erosion and gill necrosis (Declercq et al., 2013), but *F. columnare* has been isolated from fish internal organs without any external lesions (Hawke and Thune, 1992). Columnaris affects all life stages from newly hatched fry to fish that have reached the harvest stage (Panangala et al., 2007).

In aquaculture systems, fish are commonly infected by two or more pathogens (Xu et al., 2007). There is increasing evidence that co-infections contribute to the severity of some infectious diseases, especially bacterial diseases (Bandilla et al., 2006; Busch et al., 2003; Labrie et al., 2004; Pylkkö et al., 2006; Xu et al., 2007). *F. columnare* and protozoan *I. multifiliis* are two common pathogens of cultured tilapia (*Oreochromis* spp.). The temperature ranges of columnaris outbreaks (Declercq et al., 2013) overlap the optimum temperature window of *I. multifiliis* infection at 20–25 °C (Dickerson, 2012; Matthews, 2005). Supporting to this phenomena, some early studies noted difficulties in establishing columnaris by immersion challenge, perhaps requiring another infection. The previous studies utilized abrasion, temperature stress and/or feed deprivation to induce fish mortality (Bader et al., 2003; Dalsgaard, 1993; Shoemaker et al., 2003). Bader et al. (2003) suggested that abraded skin or gills of the fish predisposed fish to the early entry of microbial pathogens and subsequently increased fish mortality. There is no published information available on whether parasite infection will increase the susceptibility of tilapia to *F. columnare*. The objective of this study was to evaluate the

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susceptibility of hybrid tilapia to *F. columnare* after parasitism by *I. multifiliis*. Bacterial loads in gill and kidney were also compared between parasitized and non-parasitized fish.

2. Materials and methods

2.1. Fish and parasite

Sex reversed F1 hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) were obtained as fry from Aquasafra, Inc. (Bradenton, FL, USA) and reared to experimental size in indoor tanks at the USDA-ARS Aquatic Animal Health Research Unit, Auburn, AL. The hybrid tilapia were used as experimental animals because these fish are commonly used in intensive production in the US.

I. multifiliis was originally isolated from infected Silver dollar (*Metynnis argenteus*) obtained from a local pet shop and maintained by serial transmission on channel catfish (*Ictalurus punctatus*) held in 57 L glass aquaria as previously described (Xu et al., 2008). To culture theronts for the infection trial, heavily infected fish with maturing *I. multifiliis* were anesthetized with 150 mg L^{-1} tricaine methanesulfonate (MS-222) and rinsed in tank water. The skin was gently scraped to dislodge the parasites. Isolated trophonts were placed in a tank with 20 L water and incubated at 22–24 °C. Theronts for the infection trial were enumerated with a Sedgewick-Rafter cell.

2.2. Water quality

During the trial, dissolved oxygen (DO) and temperature in tanks were measured using a YSI 85 oxygen meter (Yellow Spring Instrument, Yellow Springs, OH). The mean ± SEM of dissolved oxygen was 6.5 ± 0.4 mg L^{-1} and the temperature was 24.6 ± 0.3 °C. The pH was 7.5 ± 0.1, ammonia was 0.35 ± 0.3 mg L^{-1}, hardness was 140.5 ± 12.1 mg L^{-1}, alkalinity was 161.6 ± 11.2 mg L^{-1} and nitrite concentration was 0.16 ± 0 mg L^{-1}, determined using a Hach CEL/890 Advanced Portable Laboratory (Loveland, Colorado).

2.3. Bacterial culture

Two isolates of *F. columnare* were used in this study: ALM-05-53 (obtained from a channel catfish) and TN-3-12 (isolated from a hybrid tilapia in Alabama). Both isolates were determined to be genomovar III by 16S rRNA gene-based RFLP typing (LaFrentz et al., in press). The isolates were inoculated in modified Shieh broth (LaFrentz and Klesius, 2009) and incubated aerobically on a shaker set at 28 °C and 175 revolutions per minute (rpm) for 24 h. Following 24 h of growth, cultures were adjusted to optical densities 0.802 (ALM-05-53) and 0.812 (TN-3-2012) at 540 nm by dilution with sterile modified Shieh broth. Following adjustment, a sample of each culture was taken and viability was tested by bacterial plate count method (in duplicate) to determine the colony forming units (CFU) present for the challenge experiment.

2.4. Experimental design

A total of 470 hybrid tilapia with an average length of 9.1 ± 1.1 cm (mean ± SD) and average weight of 12.4 ± 4.6 g were used in this trial. All fish protocols were approved by Institutional Animal Care and Use Committee at the Aquatic Animal Health Research Unit. Prior to the experiment, 10 randomly sampled tilapia were parasitologically and microbiologically examined to verify that the fish were free of *I. multifiliis* and *F. columnare* infections. Briefly, a wet mount sample was prepared from gill clips and scraping of both sides of caudal fin of each fish was done to examine for *I. multifiliis* infection. Kidney and gill tissues were aseptically removed from each fish and plated onto modified Shieh agar containing 1 μg mL^{-1} tobramycin (Decostere et al., 1997). The remaining kidney and gill were collected to verify the absence of *F. columnare* using real-time PCR (see below). All 10 sampled fish were negative for *I. multifiliis* and *F. columnare*.

A preliminary trial was conducted to determine an appropriate theront concentration for parasite infection in the trial. In the preliminary trial, three tanks of 20 tilapia were exposed to *I. multifiliis* theronts at 0, 40,000 or 80,000 theronts $fish^{-1}$ for 1 h. Fish exposed to theronts at 40,000 and 80,000 theronts $fish^{-1}$ resulted in 30% and 90% mortality, respectively. Based on these results of preliminary trial, fish were exposed to 30,000 theronts $fish^{-1}$ for 1 h in the experiment so parasite infection would result in low or no mortality.

The remaining 400 fish were first divided equally into four tanks for parasite infection. Water was lowered to 25 L in each tank prior to *I. multifiliis* theront exposure. For two tanks, *I. multifiliis* theronts were added to each tank at 30,000 theronts $fish^{-1}$ and the fish were exposed to theronts for 1 h with aeration. The fish in the other two tanks were not exposed to *I. multifiliis* theronts but kept in 25 L water for 1 h with aeration. Water flow (0.5 L min^{-1}) was resumed after 1 h and the fish were monitored for *I. multifiliis* infection in those tanks. Five days post-theront exposure, 10 fish from each tank were inspected for parasite infection level by wet mount from caudal fins under a microscope. All examined fish from the infected tanks showed *I. multifiliis* infection with 13 ± 7 trophonts per 40× magnification view (optic 10× and objective 4×), approximately 18.1 mm^2 per viewing area. The remaining 360 infected and non-infected fish were divided into 18 tanks with 20 fish per tank that received the following treatments: 1) non-infected control; 2) infected by *I. multifiliis* at 30,000 theronts $fish^{-1}$ alone; 3) infected by *F. columnare* ALM-05-53 at 4.59×10^7 CFU mL^{-1}; 4) infected by *I. multifiliis* at 30,000 theronts $fish^{-1}$ and exposed to *F. columnare* ALM-05-53 at 4.59×10^7 CFU mL^{-1}; 5) infected by *F. columnare* TN-3-2012 at 4.27×10^7 CFU mL^{-1}; and 6) infected by *I. multifiliis* at 30,000 theronts $fish^{-1}$ and exposed to *F. columnare* TN-3-2012 at 4.27×10^7 CFU mL^{-1}. To challenge with *F. columnare*, fish were immersed in water in buckets with ALM-05-53 or TN-3-2012 for 15 min. Fish not exposed to the bacterium were kept in water with Shieh broth for the same duration. After challenge the fish and challenge water were poured into the appropriate tanks and water flows were adjusted to 0.4–0.5 L min^{-1}. There were triplicate tanks in each treatment. Fish mortality was recorded and dead fish were examined for *I. multifiliis* and *F. columnare* infection twice daily for 17 days. At 3 and 6 days post-*F. columnare* challenge (8 and 11 days post-*I. multifiliis* exposure), two fish were randomly sampled from each tank to check for *I. multifiliis* infection and then gill and kidney were collected for *F. columnare* quantification. To check for *I. multifiliis* infection, two wet mount samples were prepared from skin by scraping caudal fin and two from gill filaments (5 × 5 mm) cut from the opercular cavity on both sides of each fish. Skin and gill samples were observed under a microscope and the numbers of trophonts per sample were randomly counted by 2 viewing areas at 40× magnification. *I. multifiliis* infection was assessed based on the number of trophonts in fish skin and gill per viewing area and expressed as none, <10, 10–20 and >20 trophonts per field of view. For *F. columnare* quantification by real-time polymerase chain reaction (qPCR), gill and kidney tissues were aseptically removed from each fish and stored at −20 °C until processed for DNA extraction.

2.5. Bacterial genomic DNA isolation and generation of standard curve

Pure cultures of *F. columnare* were incubated in modified Shieh broth at 28 ± 2 °C and 150 rpm in a shaker until an OD 0.802 was reached for ALM-05-53 and OD 0.812 for TN-3-12, corresponding to 4.27×10^7 CFU mL^{-1} for ALM-05-53 and 4.59×10^7 CFU mL^{-1} for TN-3-12 by the standard plate-count method. Genomic DNA (gDNA) from bacterial pellets was extracted and purified using DNeasy tissue kit following the protocol in the manufacturer's instructions (Qiagen, Valencia, CA, USA). RNase was used to remove RNA from DNA samples.

DNA yield and purity were determined spectrophotometrically using Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The purified gDNA was stored at −20 °C until used. For standards, the gDNA extracted from both *F. columnare* isolates was serially diluted (10-fold) from 5 ng to 0.5 pg mL^{-1}, which was equivalent to 1.5×10^6–1.5×10^2 genomes of *F. columnare*. The threshold cycle (Ct) value is defined as the cycle in which fluorescence is first measured. The Ct values (y-axis) are plotted against the $\log_{10}$ DNA concentrations of *F. columnare* (x-axis). The standard curve revealed a linear correlation between Ct values and log amount of nucleic acid ($Y = -3.4575X + 22.008$) with $R^2 = 0.99$ (Fig. 1).

2.6. Genomic DNA isolation from fish tissues

Each tissue was weighed (approximately 20 mg) upon thawing and recorded. The fish organ was macerated with sterilized Kontes disposable pestles in a microcentrifuge tube. Total gDNA of *F. columnare* in fish tissues was extracted by the DNeasy tissue kit and eluted with a volume of water equal to 1 μL water per mg tissue. DNA yield and purity were determined spectrophotometrically using a Nanodrop ND-1000. The purified gDNA was stored at −20 °C until used.

2.7. Quantitative real-time PCR

TaqMan-based qPCR was used for the detection and quantitation of *F. columnare* in infected fish as described by Panangala et al. (2007). The qPCR has been demonstrated to have high specificity and sensitivity to quantify *F. columnare* in fish tissues, such as blood, gills and kidney (Panangala et al., 2007). The qPCR was performed on an Applied Biosystems 7500 Real-Time PCR machine (ABI, Foster City, CA) using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA). Two *F. columnare*-specific primers (forward 5′-CCTGTACCTAATTGGG GAAAAGAGG-3′ and reverse 5′-GCGGTTATGCCTTGTTTATCATAGA-3′) and a dual-labeled probe (5′-ACAACAATGATTTTGCAGGAGGAGTATCTG ATGGG-3′) were used for specific detection of *F. columnare*. The probe was labeled at the 5′ end with the fluorescent reporter dye [6-carboxy fluorescein (FAM) with an emission spectrum of 518 nm] and at the 3′ end with the fluorescent quencher dye [6-carboxy-N,N,N′,N′-tetramethylrhodamine (TAMRA) with an emission spectrum of 582 nm] (Panangala et al., 2007). The primers targeted a 113 bp nucleotide region of the chondroitin AC lyase gene of *F. columnare* (GenBank accession number AY912281; Panangala et al., 2007; Xie et al., 2005). The qPCR mixture in a final volume of 12.5 μL consisted of 1 μL of gDNA from tissue samples, 0.5 μL of 5 mM forward primer, 0.5 μL of 5 mM reverse primer, 0.25 μL fluorogenic probe and 10.25 μL of 1× Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA). Reactions were performed using the following conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Extracted DNA from fish tissue (1 μL) was used as template in qPCR and the DNA concentration of *F. columnare* in fish tissue was determined via the standard curve. Since 1 μL of eluted sample was run in qPCR, the amount of bacterial DNA in each mg of tissue was equal to bacterial DNA concentration (pg μL^{-1}) × eluted volume/tissue weight (mg). Bacterial DNA in each mg of tissue was calculated as genome equivalents per mg of tissue (GEs mg^{-1}) based on the genome size of *F. columnare* = 3.2336 fg $cell^{-1}$ (Tekedar et al., 2012).

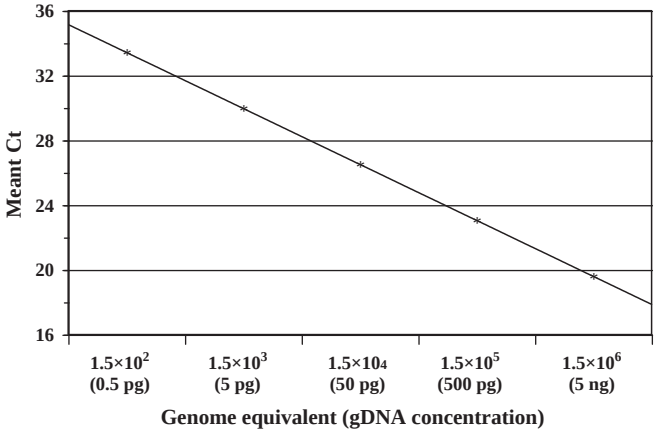

Fig. 1. Standard curve for quantifying *F. columnare* in hybrid tilapia. The Ct values (Y) are plotted against genome equivalent ($\log_{10}$ DNA concentrations) of *F. columnare* (X), $Y = -3.4575X + 22.008$ ($R^2 = 0.99$).

2.8. Statistical analysis

Data were analyzed with SAS software (SAS Institute, 1989). Mean days to death (MDD) were calculated by Lifetest procedure (Kaplan–Meier method). Mortalities and the levels of *F. columnare* DNA in fish (GEs mg^{-1}) from different treatment groups were compared with Duncan multiple range tests. *P*-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Parasite prevalence and infection level

No parasites were observed on fish that were not exposed to theronts. All fish were infected by the parasite after exposure to 30,000 theronts $fish^{-1}$ (Table 1). Both skin and gills of infected fish showed an infection level with less than 10 trophonts per 40× view (4× objective and 10× optic) 8 days post-exposure (dpe) to *I. multifiliis* (3 dpe to *F. columnare*). No parasites were observed both from the skin and gill 11 dpe to *I. multifiliis* (6 dpe to *F. columnare*).

3.2. Infection of fish with F. columnare

No *F. columnare* was isolated from either parasitized or non-parasitized tilapia that were not exposed to bacteria regardless of isolate ALM-05-53 or TN-3-2012. On the other hand, *F. columnare* was isolated from 33–50% of non-parasitized tilapia and 83–100% from parasitized tilapia at 3 dpe to *F. columnare* (Fig. 2). Among *F. columnare* positive fish, *F. columnare* was detected in 53% of the fish, both the gill and the kidney being positive (data not shown). In the remaining 47% of fish positive for *F. columnare*, the bacterium was detected in gill alone or kidney alone 3 dpe to the bacterium. Prevalence of *F. columnare* was significantly higher in parasitized fish than non-parasitized fish 6 dpe to *F. columnare* ALM-05-53 or TN-3-2012.

Table 1
Prevalence and infection level of *Ichthyophthirius multifiliis* in hybrid tilapia 8 days post-exposure to *I. multifiliis* (3 days post-exposure to *F. columnare*). Skin and gill samples were observed under a microscope and the *I. multifiliis* infection was expressed as the numbers of trophonts per viewing area at 40× magnification. No parasites were observed in the skin and gill 11 days post-exposure to *I. multifiliis* and data were not present in the table.

Theronts/fish	*F. columnare* isolate	Skin		Gill	
		Infection (%)	Prevalence level	Infection (%)	Prevalence level
0	No	0	0	0	0
0	ALM-05-53	0	0	0	0
0	TN-3-2012	0	0	0	0
30,000	No	66.6	<10	100.0	<10
30,000	ALM-05-53	100.0	<10	100.0	<10
30,000	TN-3-2012	83.3	<10	100.0	<10

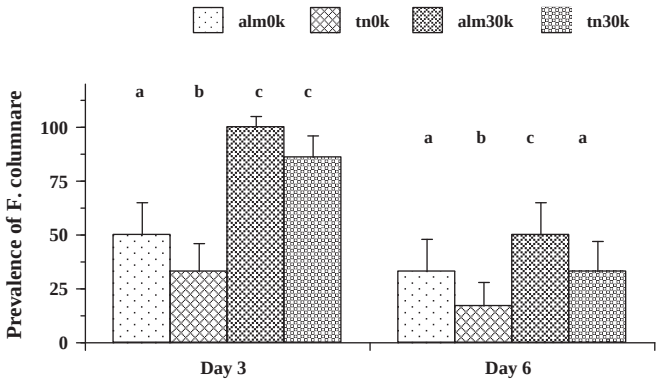

Fig. 2. Prevalence of *F. columnare* was significantly lower in hybrid tilapia not exposed to *Ichthyophthirius multifiliis* theronts (0 k) than fish exposed to 30,000 theronts fish^{-1} (30 k) at 3 and 6 days post-exposure to *F. columnare* ALM-05-53 (alm) or TN-3-2012 (tn). Within a given sampling day, means with different superscript letters are statistically different ($P < 0.05$).

3.3. Fish mortality after exposure to F. columnare

The fish showed 2.1% mortality when infected by *I. multifiliis* alone at 30,000 theronts fish^{-1} (Table 2). The fish yielded no mortality when challenged with *F. columnare* TN-3-2012 alone and 29.1% mortality when challenged with *F. columnare* ALM-05-53 alone. On the other hand, the parasitized fish showed 25.0% and 60.4% mortalities after exposure to *F. columnare* TN-3-2012 and ALM-05-53, respectively. Mortalities were significantly higher in *I. multifiliis* parasitized fish than those in non-parasitized fish ($P < 0.05$) after exposure to *F. columnare*. Majority of fish mortality occurred in the first 24–48 h post-exposure to *F. columnare* with MDD ≤ 1.5. Clinical signs at first 48 h included mucus sloughing, skin darkness and rapid opercular movement. Twenty-five percent of fresh dead fish following *F. columnare* exposure were cultured to confirm the presence of *F. columnare* as the cause of mortality. Results demonstrated that all dead fish were culture positive from the gill and kidney samples.

3.4. Load of F. columnare in fish

F. columnare in fish tissues was quantified by qPCR and reported as genome equivalents per mg of tissue (GEs mg^{-1}). No *F. columnare* was detected in tissues of parasitized or non-parasitized fish prior to exposure to *F. columnare*. The bacterial loads increased significantly ($P < 0.05$) in the gill of parasitized fish compared to those of non-parasitized fish after exposure to *F. columnare*. The bacterial load in gill of parasitized fish (5702.5 GEs mg^{-1}) was 14 fold higher than that of non-parasitized fish (415.4 GEs mg^{-1}) 3 dpe to *F. columnare* ALM-05-53 (Table 3). The parasitized fish showed a bacterial load of 2526.1 GEs mg^{-1} which was 13 fold higher than that of non-parasitized fish (197.0 GEs mg^{-1}) 3 dpe to *F. columnare* TN-3-2012. Similarly, the bacterial loads in the gill of parasitized fish were 5 fold and 14 fold higher compared to those of non-parasitized fish 6 dpe to *F. columnare* TN-3-2012 and ALM-05-53, respectively (Table 3).

Table 2
Cumulative mortality of hybrid tilapia parasitized with *Ichthyophthirius multifiliis* after challenge using *F. columnare* ALM-05-53 or TN-3-2012. Fish mortality (±SEM) was the mean of 48 fish in 3 tanks and observed for 21 days post-exposure to *F. columnare* by immersion for 15 min. Within a given column, means followed by different superscript letters are statistically different ($P < 0.05$).

Theronts fish^{-1}	*F. columnare* isolate	Fish number	Dead number	Mortality (%)	MDD[1]
0	No	48	0	0 ± 0^{a}	NA[2]
30,000	No	48	1	2.1 ± 2.1^{a}	5.0
0	ALM-05-53	48	14	29.1 ± 12.7^{b}	1.0
30,000	ALM-05-53	48	29	60.4 ± 22.0^{c}	1.5
0	TN-3-2012	48	0	0 ± 0^{a}	NA
30,000	TN-3-2012	48	12	25.0 ± 9.5^{b}	1.3

[1]Mean day to death; [2]not available.

Table 3
The genome equivalent of *F. columnare* (±SEM) in tissues of hybrid tilapia (GEs mg^{-1}) exposed to no *I. multifiliis* theronts or 30,000 theronts fish^{-1} at day 3 and day 6 post-exposure to different *F. columnare* isolates. Within a given sampling day and a fish organ, means followed by different superscript letters are statistically different ($P < 0.05$).

Tissues	Theronts fish^{-1}	*F. columnare* isolate	Day 3	Day 6
			GEs mg^{-1}	GEs mg^{-1}
Gill	0	ALM-05-53	415.4 ± 28.2^{a}	191.3 ± 7.4^{a}
	0	TN-3-2012	197.0 ± 13.3^{a}	49.4 ± 3.3^{b}
	30,000	ALM-05-53	5702.5 ± 172.4^{b}	2649.7 ± 160.5^{c}
	30,000	TN-3-2012	2526.1 ± 120.2^{c}	236.3 ± 12.4^{a}
Kidney	0	ALM-05-53	471.8 ± 18.1^{a}	0 ± 0^{a}
	0	TN-3-2012	83.5 ± 8.3^{b}	0 ± 0^{a}
	30,000	ALM-05-53	8180.1 ± 19.4^{c}	0 ± 0^{a}
	30,000	TN-3-2012	1586.6 ± 24.4^{a}	0 ± 0^{a}

The kidneys of parasitized fish showed significantly higher bacterial loads ($P < 0.05$) than those of non-parasitized fish after exposure to *F. columnare* (Table 3). The bacterial load in kidney (8180.1 GEs mg^{-1}) of parasitized fish was 17 fold higher than that of non-parasitized fish (471.4 GEs mg^{-1}) 3 dpe to *F. columnare* ALM-05-53. When exposed to *F. columnare* TN-3-2012, the bacteria in kidney of parasitized fish (586.6 GEs mg^{-1}) was 7 fold higher than those of non-parasitized fish (83.5 GEs mg^{-1}). No *F. columnare* was detected by qPCR in kidney at 6 dpe to *F. columnare*.

4. Discussion

The loads of *F. columnare* detected by qPCR increased significantly in gills and kidney of parasitized fish compared to those of non-parasitized fish. Parasite infection by *I. multifiliis* resulted in damage to the fish skin and gills. When theronts move between epithelial cells to seek sites for adherence, the burrowing theronts push epithelial cells apart and cause cell injury. The growing trophonts expand to occupy tissue space and destroy adjacent cells (Xu et al., 2000). Extensive cell necrosis and histolysis occur around trophonts in the epithelium (Hines and Spira, 1974; Ventura and Paperna, 1985). The mucus and epidermis are the first line of defense in fish and act as protective shields against invasive microorganisms. Fish mucus contains a variety of antimicrobial compounds such as antibacterial peptides, lysozyme, proteases, and antibodies that may protect underlying epidermal cells from bacterial adhesion and colonization (Soto et al., 2008). It has been demonstrated that parasite infections can disrupt the first line of defense by causing abrasions, lesions and ulcers in the skin and gills, thereby creating putative routes for bacterial invasion (Cusack and Cone, 1986; Hines and Spira, 1974; Kanno et al., 1990; Labrie et al., 2004) and increased susceptibility to bacterial pathogens (Bandilla et al., 2006; Busch et al., 2003; Pylkkö et al., 2006). The present study further demonstrated that parasitic infection enhanced bacterial invasion, resulted in high loads of bacteria in fish tissues and subsequently increased fish mortality.

Tilapia exhibited higher bacterial loads in gill and kidney from fish exposed to *F. columnare* ALM-05-53 than those exposed to *F. columnare* TN-3-2012. The bacterial loads were 2–5 fold higher in gill and kidney of fish 3 dpe to *F. columnare* ALM-05-53 compared to those of samples obtained from fish exposed to TN-3-2012. Similar results were noted in fish mortality as fish exposed to *F. columnare* ALM-05-53 showed significantly higher mortality than fish exposed to *F. columnare* TN-3-2012. Virulence differences have been demonstrated among isolates of *F. columnare* (LaFrentz et al., 2012; Shoemaker et al., 2008). In this study, both isolates were genomovar III suggesting intra-genomovar virulence differences in *F. columnare*. Suomalainen

et al. (2006) demonstrated a similar result when studying genomovar I *F. columnare* isolates in rainbow trout. They further suggested that the isolates showing differential mortality could be identified (subtyped) by automated ribosomal intergenic spacer analysis (ARISA). The results in this study demonstrated that parasitic infection enhanced bacterial invasion and increased mortality with both genomovar III *F. columnare* isolates.

Tilapia infected by *I. multifiliis* alone at 30,000 theronts fish^{-1} showed low mortality and died with a MDD of 5 days. The water temperature was maintained at 24 °C during this trial. At this temperature, *I. multifiliis* requires 5–6 days to complete its life cycle. Mature trophonts usually leave infected fish and multiply in tank water into infective theronts. The theronts from the second life cycle of *I. multifiliis* reinfect the fish and cause mortality if the theront numbers become high in water. The MDD is usually 5 days or greater if fish die of *I. multifiliis* infection. In this trial, tilapia mostly died with a short MDD (≤1.5 days) when *I. multifiliis* parasitized fish were exposed to *F. columnare* ALM-05-53 or TN-3-2012. The acute mortalities in this trial are similar to those reports in tilapia following *F. columnare* challenges (Amin et al., 1988; Kuo et al., 1981). The light load of parasite *I. multifiliis* was not a primary contributor to mortality, but served as an enhancer for the subsequent bacterial infection. In a previous study, tilapia were exposed to 40,000 theronts fish^{-1} overnight in order to achieve 50% or higher mortality (Xu et al., 2008). In this study, tilapia exposed to 30,000 theronts fish^{-1} for 1 h had low mortality (<3%). However, the parasitism of fish enhanced invasion of *F. columnare* and greatly increased fish mortality after exposure to either *F. columnare* ALM-05-53 or TN-3-2012 isolate. The exact relationship between the parasite and *F. columnare* needs further study. This study suggests that damage of the fish skin and gills by the parasite created routes for bacterial invasion. It is also possible that *I. multifiliis* serves as a vector to transfer *F. columnare* into fish. Sun et al. (2009) demonstrated the presence of *F. columnare* DNA in tomonts and theronts and the authors suggested that the parasite may serve as a carrier of *F. columnare*. Similarly, the theronts and trophonts from *I. multifiliis* have been demonstrated to vector and transmit *Edwardsiella ictaluri* in channel catfish (Xu et al., 2012, 2013). Since *F. columnare* are external bacteria of fish, further studies are needed to closely examine the vector ability of the parasite to carry *F. columnare*.

5. Conclusions

I. multifiliis-parasitized tilapia showed higher mortality when co-infected with *F. columnare* than non-parasitized fish. The loads of *F. columnare* in gill and kidney of *I. multifiliis*-parasitized fish were significantly (≥10 fold) higher than those of non-parasitized fish. This work suggests that prevention of parasite infection in fish will not only reduce the direct damage caused by the parasite but will also reduce fish mortality due to bacterial co-infection.

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